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PRINCIPAL INVESTIGATOR: Roger W. Giese, Ph.D.

CONTRACTING ORGANIZATION: Northeastern University
Boston, Massachusetts 02115

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13. ABSTRACT *(Maximum 200 words)*

The purpose of this project is to optimize and apply new methodology for the detection of unknown DNA adducts in breast and, for comparison, other human tissues. In this methodology the DNA adducts are labeled with a fluorescent dye to enhance their detection. Although the first dye tested (IMI1) was not fully stable, this problem was overcome by preparing IMI2. High dye purity is required, and this was accomplished for IMI2 by means of flash chromatography/submarine gel electrophoresis, which brought the purity to 99.9998%. A basic protocol has been set up for the entire method, starting with human cell culture or human autopsy tissue samples, including breast samples and finishing with detection by capillary electrophoresis with laser induced fluorescence detection (CE-LIF). Standard adducts, after labeling with IMI2, were detected by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), with 300 fmol in the laser beam. Thus, the methodology is emerging as planned.

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Roger Grais 7/22/98

PI - Signature Date

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INTRODUCTION

DNA adducts, the consequence of covalent damage to DNA as by toxic chemical or physical conditions, play an important role in carcinogenesis and mutagenesis (1, 2). This is because DNA is an ultimate target in the body for agents causing these events. Thus it is important to measure DNA adducts comprehensively and accurately in human tissue as a way to learn more about the origins of disease processes having a genetic component such as human breast cancer.

DNA adducts are measured currently both as known and unknown compounds. The methodology available for measuring known DNA adducts, while not perfect, is adequate in many cases. However, current methodology for detecting unknown DNA adducts has severe shortcomings. Basically what is available for this purpose is ^{32}P-postlabeling, involving separation of the adducts, once they are ^{32}P-postlabeled, by thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). The problems with ^{32}P-postlabeling for detecting unknown DNA adducts are as follows. First, unknown adducts are labeled to different, unknown degrees, so there is no guarantee that all the adducts are detected, nor even whether the more prominent spots or peaks represent the prominent adducts. Second, for unknowns, the method has been applied only to bulky adducts, since these are the easiest to detect. Third, because of the radioactivity, the separation techniques employed are kept simple, which in turn limits the resolution and characterization. Fourth, the limited resolution can make it difficult to compare results from different laboratories for unknown adducts. Finally, the method does not identify unknowns except by comparison with known adducts.

Sorting out the origins of breast cancer may require the detection and characterization of unknown DNA adducts. Thus we have set out in this project to develop improved methodology for detecting unknown DNA adducts in breast tissue. As a starting point, taking into account the information cited below, and to provide a comparison with data from ^{32}P-postlabeling, we will first apply the method to bulky DNA adducts in breast tissue. Subsequently the methodology can be expanded to give comprehensive detection of unknown DNA adducts.

The concept of the new methodology is the same as that of ^{32}P-postlabeling, but the details are quite different. A fluorescent tag is used in place of ^{32}P, chemical rather than enzymatic labeling is done, and capillary electrophoresis is used as a supplement to an HPLC separation to achieve high resolution. Final detection results from laser-induced fluorescence rather than radioactivity.

In principle, this overcomes or mitigates all of the above limitations of ^{32}P-postlabeling. It helps indirectly to characterize the unknowns since, once an interesting adduct is found, it can be scaled up and put into a mass spectrometer as a fluorescently-labeled product. In most laboratories, including ours, one would never put a ^{32}P-postlabeled compound into a mass spectrometer. At an earlier stage in our project we demonstrated the detection by mass spectrometry of a deoxynucleotide labeled with our fluorescent dye.

Little is known about DNA adducts and breast cancer, although it is generally considered that they must play a major role in this disease (3-5). Malins and coworkers have studied oxidative damage to DNA in both normal and cancerous human breast tissue (6). They found that the latter tissue contains a higher concentration of the oxidative adducts 8-oxoadenine, 8-oxoguanine, and 5-hydroxymethyluracil. Also the ratio of the 8-oxoadenine product to the corresponding ring-opened purine (which can form reductively from the 8-oxoadenine product) was higher in cancerous *vs.* normal tissue. This latter observation is consistent with the production of elevated levels of hydrogen peroxide by tumor cells (7). The amounts of these oxidative adducts in normal breast tissue were reported to be about 1-5 adducts in 10^4 normal nucleotides. Other adducts in biological samples in general are encountered at lower levels, e.g., 1 in 10^7 - 10^8 nucleotides (1,2).

Some other, more general observations may be relevant as well to a relationship between DNA adducts and breast cancer: the increasing incidence of this disease (5); its promotion (in rodents) by steroid hormones (4); the occurrence of genetic events such as oncogene activation in breast cancer (8); the existence of certain risk factors such as exposure to ionizing radiation and alcohol use (5); the ability of environmental xenobiotics such as polyaromatic hydrocarbons to induce mammary cancer in rodents (9) coupled with the fact that such agents tend to accumulate in human breast fat (10); and the increased incidence of mammary tumors in rodents on a high fat diet coupled with a suggested correlation between a human diet rich in fat (and protein) and higher DNA content of breast cancer cells (11).

EXPERIMENTAL

Equipment. The submarine gel electrophoresis system was model EC370 from E-C Apparatus Corporation (Holbrook, NY). The capillary electrophoresis laser-induced fluorescence (CE-LIF) equipment was described before (12). A SpeedVac Concentrator from Savant Instruments Inc. (Holbrook, NY) was used for vacuum drying and concentration (without heating). The glassware was used as obtained.

Reagents. Methanol, methylene chloride, tetrahydrofuran and ethyl acetate were HPLC Solvent Grade from J.T. Baker. MES buffer (10 mM, pH

6.0) was prepared fresh by combining 475 mL water, 25 mL methanol, 1.066 g 2-[N-morpholino]ethanesulfonic acid (Sigma, St. Louis, MO), and adjusting the pH with 1 N sodium hydroxide. Any over-adjustment was corrected with 1 M sulfuric acid. Sucrose solution was 3 g (Sigma) in 10 mL of MES. Ni-NTA-Agarose was from QIAGEN (Valencia, CA). Histamine-methanol solution was prepared by combining 50 mg of histamine (No. 53290 from Fluka, Milwaukee, WI) and 100 µL of methanol. Other reagents were obtained as described (12).

IMI2 Synthesis. BODIPY® FL SE (1 mg, Molecular Probes, Eugene, OR) was dissolved in 200 µL of freshly distilled (over sodium) tetrahydrofuran, and 10 µL of histamine-methanol solution was added, giving an immediate precipitate. After 20 standing in the dark, the reaction mixture was transferred to a 10-mL test tube and 1 mL water was added, dissolving the precipitate. Two portions of 1 mL of freshly distilled methylene chloride were used to extract the product, and these extracts were combined, dried in a SpeedVac, and stored in the dark at 4°C prior to purification as follows by silica flash chromatography and submarine gel electrophoresis.

Silica Flash Chromatography. A silica column was prepared by packing Silica Gel 60 (particle size 0.040-0.063 mm, EM Science, Gibbstown, NJ) in a 10-mL pipette (up to the 10-mL mark) plugged with glass wool. The mobile phase was 25:75 methanol:ethyl acetate, v/v with 0.05% triethylamine (prepare 100 mL). After the column was pre-washed with 20 mL of mobile phase, the sample was dissolved in 50 µL methanol followed by 150 µL of mobile phase and applied. The major (slower) component was collected and dried in a SpeedVac after the eluent was divided into two 2-mL glass vials (No. 60910L with rubber lined cap from Kimble Glass).

Submarine Gel Electrophoresis. Ultrapure Agarose (0.5 g from Gibco, Grand Island, NY) was dissolved in 50 mL of MES buffer with boiling, and poured into the 4-comb plate after cooling to 60-70° (cool enough to handle). The plate was kept at room temperature for 2 hours to form the gel, and the comb was removed carefully. The dimensions of the gel were 82 x 67 x 10 mm (l x w x h). After the gel plate was placed into the apparatus, MES buffer was added to a level 1-mm above the gel. IMI2 (ca 0.5 mg) was dissolved in 80 uL of methanol, and 200 uL of sucrose solution was added. The sample solution was loaded quickly into the bottom of the four wells, and a current of 50 mA was established by applying 250 V. The electrophoresis was run without cooling for 40 in the dark, and the voltage was adjusted every 10 as needed to keep 50 mA. The gel was removed, rinsed with 50 mL of water, and the tailed dye band, that had traveled 6 cm, was sliced out with a water-washed razor blade, giving a 67 x 10 x 10 mm gel slab that was sliced further (20 pieces), placed in a 20 mL disposable scintillation vial (no. 986741 with polypropylene cap from Wheaton), covered with 7 mL of methanol (to extract the dye), and stored at 4°C in the dark overnight, or for at least 2 h.

IMAC of IMI2 to Assess Impurities. A column was prepared by packing 500 µL of Ni-NTA-Agarose gel suspension in a 5.75 in. long capillary Pasteur pipette plugged with a 0.6 cm^2 piece of Kimwipe (Fisher Scientific, Pittsburgh, PA) and washed with one column volume of 10% methanol. Aqueous sample of IMI2 (see figure caption) was loaded into the bed with air pressure. Elution was done by forcing 2 x 50 µL of 10% methanol through the column with air pressure. The collected sample was concentrated in a SpeedVac to 15 µL (volume measured by uptake into a pipet) before injection of an aliquot into CE-LIF.

Capillary Electrophoresis. CE was performed in a 70 cm long fused-silica capillary (75 µm i.d., Polymicro Technologies, Inc., Phoenix, AZ) with the detection window 50 cm from the anode injection end. Samples were introduced in the capillary hydrodynamically by raising the anode end 10 cm higher for 20 sec. Applying 16 kV to the capillary gave 35 µA. The electrolyte was 10 mM pH 10.6 sodium carbonate buffer, 50 mM sodium dodecylsulphate.

RESULTS AND DISCUSSION

A. Purification of labeling dye

Detection of trace analytes by labeling with a fluorophore tends to require post-derivatization sample cleanup prior to detection in order to overcome interferences. These interferences have three origins: residual fluorophore, contaminants in the fluorophore including fluorophore decomposition products, and fluorescent side products formed in the reaction. With IMI dyes, the first type of interference can be minimized by using immobilized metal affinity chromatography (IMAC) at the end of the labeling reaction to remove residual dye (13), and the last is reduced by the inherent specificity of the labeling reaction for phosphomonoesters (12). Thus we directed our attention, as described here, to the second problem: purifying an IMI dye to a high degree. For this work we selected IMI2, a dye which incorporates an amide as opposed to the hydrazide linkage in IMI1 (previously named "BO-IMI"), since the hydrazide linkage in the latter compound is susceptible to hydrolysis (14). No hydrolysis of the amide linkage in IMI2 was observed even when this dye was kept at room temperature in buffers with pH's ranging from 6 to 10 for two days (data not shown).

The starting point for the ultrapurification of IMI2 was dye that had been subjected to silica flash chromatography to reach a purity level in terms of fluorescence of 99.5% based on an analysis by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). A variety of chromatographic (five types of HPLC columns including mobile phase variations) and partitioning procedures (details not presented), along with immobilized metal affinity chromatography (IMAC), alone and in combination, failed to extend the purity much further. Throughout this work, it was important to minimize exposure of the dye to light and to avoid evaporation to dryness, since these conditions could increase the impurities.

We decided to try preparative electrophoresis for purification as a more orthogonal technique relative to the ones already tried, and selected a submarine gel for this purpose because of its convenience. Indeed, we estimated that a fluorescence purity of 99.9998% was achieved for IMI2 in this way. The data leading to this conclusion is shown in Figure 1. We subjected one aliquot of this reagent directly to CE-LIF (Fig. 1A, peak area 1.343×10^9), and a second aliquot first to IMAC (to selectively remove the dye) prior to evaporative concentration followed by CE-LIF (Fig. 1B). In a separate experiment, we observed that IMAC alone had a negligible impact on the purity of IMI2 at this stage (data not shown). Thus, the observed impurities are enriched 5000 times in A relative to B. We arbitrarily assumed that the observable impurities (Fig. 1B; peak areas sum to 2689) had a fluorescent brightness equivalent to that of IMI2 in estimating the fluorescence purity level. One of the peaks (e.g. the tallest one) in Fig. 1B may be residual IMI2, in which case the fluorescent purity of the dye is higher than 99.9998%. Presumably the level of nonfluorescent impurities is low, given the multiple purification steps and the high fluorescence purity. Consistent with this, the dye gives a single spot by silica TLC with UV-quench detection (developed with methanol: CH_2CL_2, 1:10, v/v), in fact, both before and after the electrophoresis purification step.

B. Standard DNA adduct detection by IMI labeling/CE-LIF

We reacted 5'-dAMP with benzo[a]pyrene diolepoxide to obtain some standard DNA adducts. The product mixture, after sample cleanup on a C18-Si cartridge, was labeled with IMI2 prior to analysis by capillary electrophoresis with laser induced fluorescence detection (CE-LIF). Also a blank sample (no 5'-dAMP) was analyzed. This led to the electropherograms shown in Fig. 2. As seen, two types of CE conditions were utilized: MEKC and non-MEKC (MEKC = micellar electrokinetic chromatography). Apparently the two major peaks are the N7G and N^2G adducts, based on their migration characteristics and the literature. These results demonstrate the high selectivity available from CE-LIF for this kind of analysis.

C. Lymphocyte DNA adduct detection by IMI2 labeling/CE-LIF

As summarized in Fig. 3, our method was able to detect a DNA adduct in human lymphocytes exposed in cell culture to benzo[a]pyrene. In this experiment we broadened the method to encompass a biological sample.

D. Tissue sample testing

As shown in Figs. 4 and 5, different electropherograms were obtained when our IMI2 labeling/CE-LIF technique was applied to DNA isolated from autopsy human tissue samples. These tissue samples were obtained from a 90-year-old woman. Of the two breast samples analyzed (Fig. 4C and Fig. 5), the latter showed many more peaks, and came from a sample containing much more fat. As shown on the right side of Fig. 5, ordinary phospholipid gives a peak at the edge of the collection of tissue sample peaks. While the

differences in the two breast samples might be considered to be consistent with the hypothesis of our project, we view the data as very preliminary and worry that it may be just an artifact. What is demonstrated in this experiment is that we have now fully broadened the method to encompass tissue samples, and obtained some results that may be interesting. Further experiments are needed to sort this out.

E. Mass Spectrometry

We have found that IMI2-labeled deoxynucleotides and representative DNA adducts can be readily detected by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS). Shown in Fig. 6 is the detection of the four normal deoxynucleotides as IMI2 conjugates in this way, and similar detection of IMI2 conjugates of benzo[a]pyrene diolepoxide DNA adducts of adenine and guanine deoxynucleotides are shown in Fig. 7 and 8, respectively. The amount of samples in the laser beam is about 300 fmol, and the S/N is $\geq$ 10 for the pseudomolecular ions. Importantly, the boost in mass contributed by the IMI2 moiety places the pseudomolecular ions in a relatively clean region of the mass spectrum. As seen in Fig. 9, the observed and calculated isotopic cluster for the pseudomolecular ion of IMI2-dAMP match up exactly. The isotopic distribution is enriched by the presence of the boron atom, which exists naturally as 19.9% ^{10}B, 80% ^{11}B. These results establish that suitably scaled up DNA adducts, after labeling with IMI2, can be directly tested by mass spectrometry.

F. Problems

Hyperpurifying IMI2 dye was a challenge that we overcame after much effort, as reported above in A. This type of problem is of general importance, and our approach to solving it has been accepted as a manuscript for publication in *Analytical Chemistry*.

CONCLUSIONS

We have advanced our new analytical methodology by preparing a more stable IMI dye (IMI2), hyperpurifying this dye, setting up sample cleanup steps that allow us to start with biological samples including human autopsy tissues, and analyzing standard IMI2-labeled DNA adducts by mass spectrometry. Thus we conclude that our methodology should continue to be developed, since it appears that its intended usefulness and advantages will be realized. In the next stage we will better define what sensitivity it can provide for biological samples, fine-tune the prototype procedure that we have developed, and apply it further to human autopsy tissue samples.

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Figure Captions

Fig. 1 Electropherograms of (A) IMI2 and (B) trace impurities in IMI2. For A, 6.0×10^{-5} M IMI2 in water was diluted with water to 8.0×10^{-8} M prior to injection. For B, 100 µL of 6.0×10^{-5} M IMI2 was subjected to IMAC, vacuum-concentrated to 15 µL, and then injected. IMI2 concentration was determined by UV, using the data established for BODIPY® FL SE (Catalog Number D-2184, ϵ = 80,500 at 502 nm in methanol, Lot Data, Molecular Probes, Eugene, OR).

Fig. 2 Electropherograms showing the detection by CE-LIF of IMI2-labeled DNA adducts arising from the reaction of benzo[a]pyrene diolepoxide with 5′-dAMP.

Fig. 3 Electropherograms showing the application of the IMI postlabeling method to human lymphocytes exposed in cell culture to BPDE. The exposure samples were prepared for us by William Thilly at MIT.

Fig. 4 Electropherogram obtained by extracting the DNA from a human autopsy breast sample with phenol/chloroform after proteolytic digestion, and then conducting steps 3-6 of Fig. 3.

Fig. 5 Electropherograms on the left were obtained as described in Fig. 4. The electropherograms on the right are from triplicate labeling and detection of egg yoke phosphatidic acid.

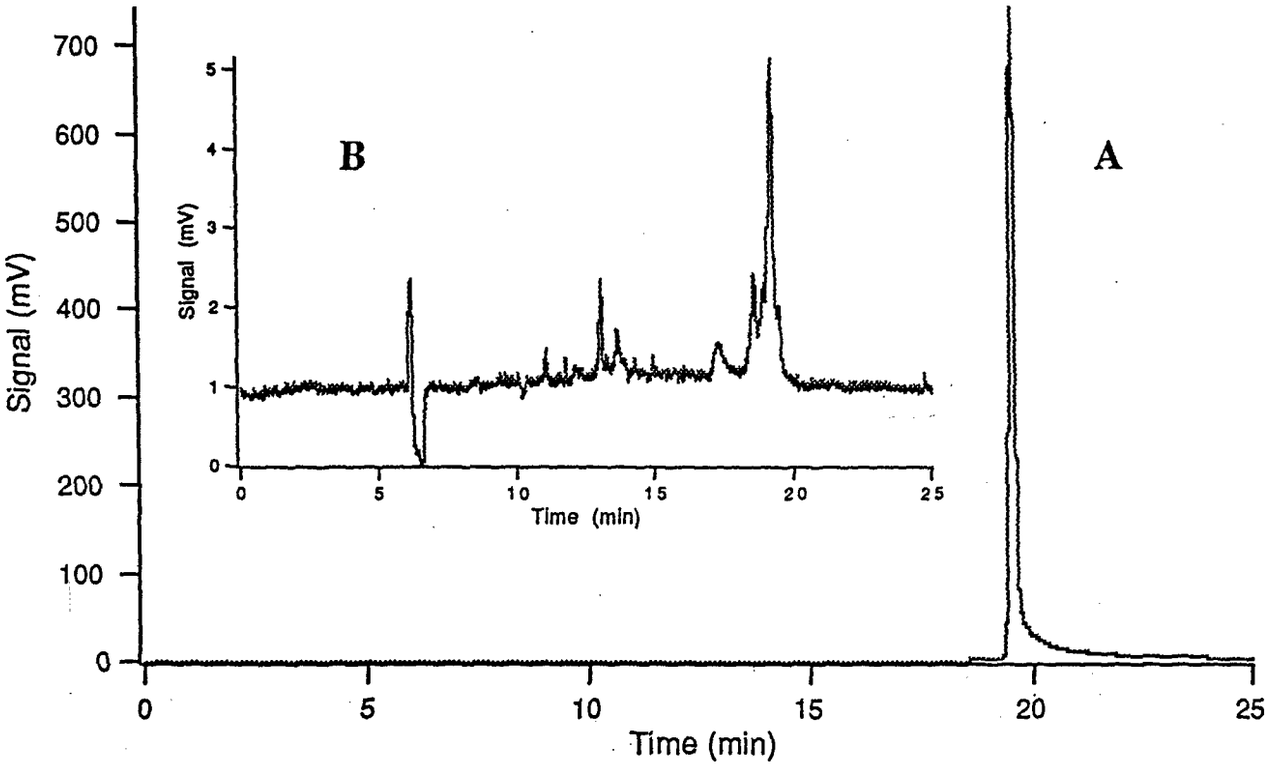
Fig. 6 Detection of IMI2-labeled 5′-deoxynucleotides by MALDI-MS.

Fig. 7 Detection of an IMI2-labeled benzo[a]pyrene DNA adduct of 5′-dAMP by MALDI-MS.

Fig. 8 Detection of an IMI2-labeled benzo[a]pyrene DNA adduct of 5′-dGMP by MALDI-MS.

Fig. 9 Observed (left) and calculated (right) isotopic distribution for the pseudomolecular ion $(M-H)^-$ of IMI2-labeled dAMP by MALDI-MS.

Figure 1



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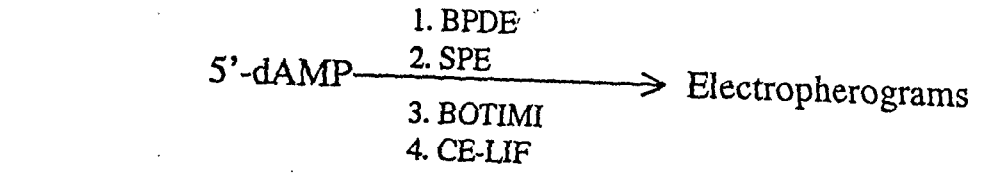

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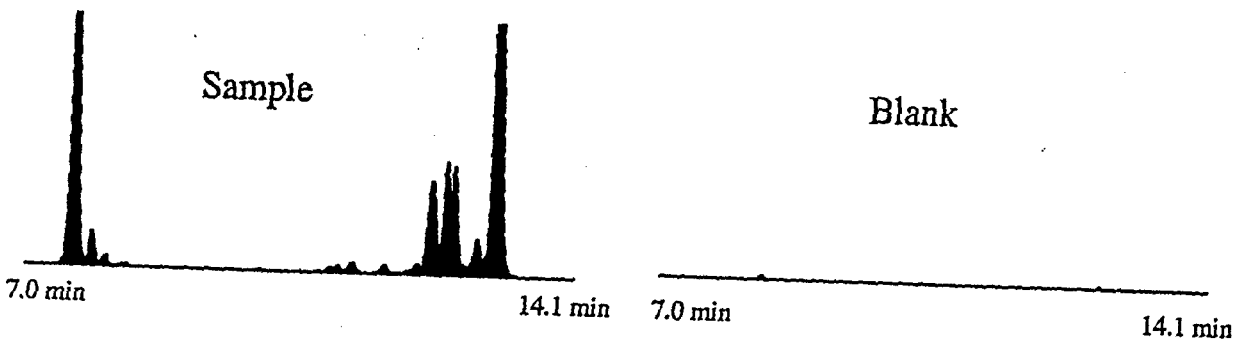

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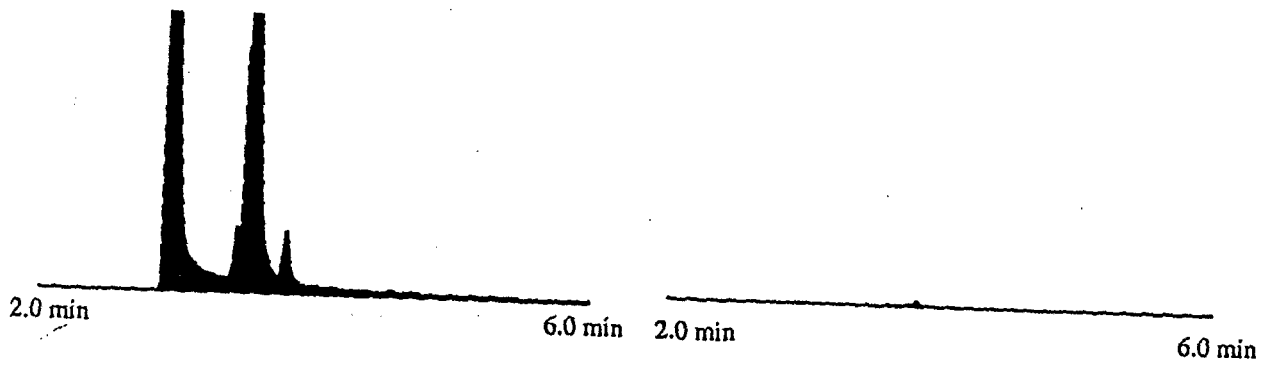


Human Lymphocytes

1. BPDE (W. Thilly)
2. Qiagen Protocol
3. Nuclease P1
4. SPE (Waters Oasis)
5. BOTIMI
6. CE-LIF (MEKC)

→ Electropherogram

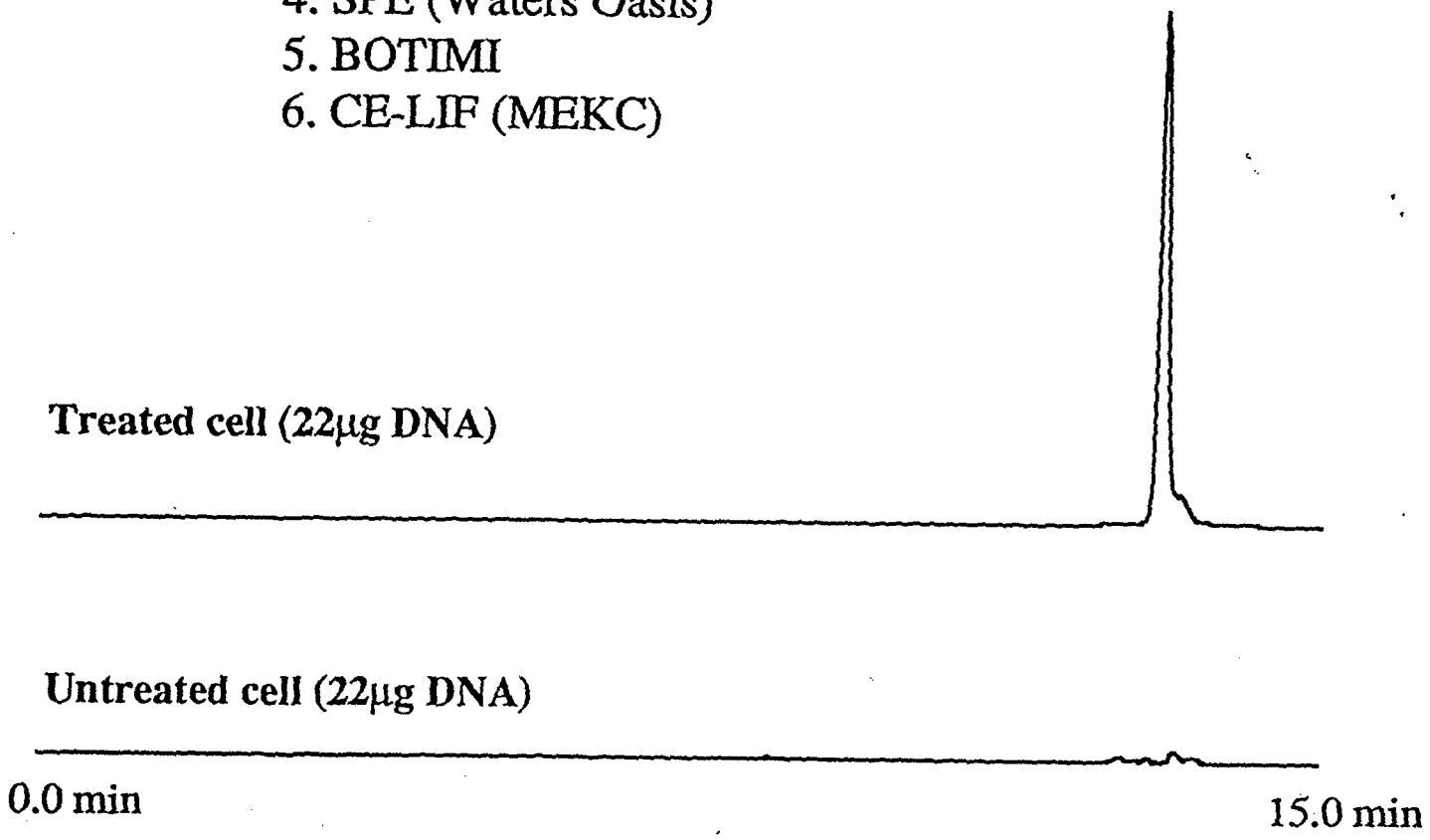
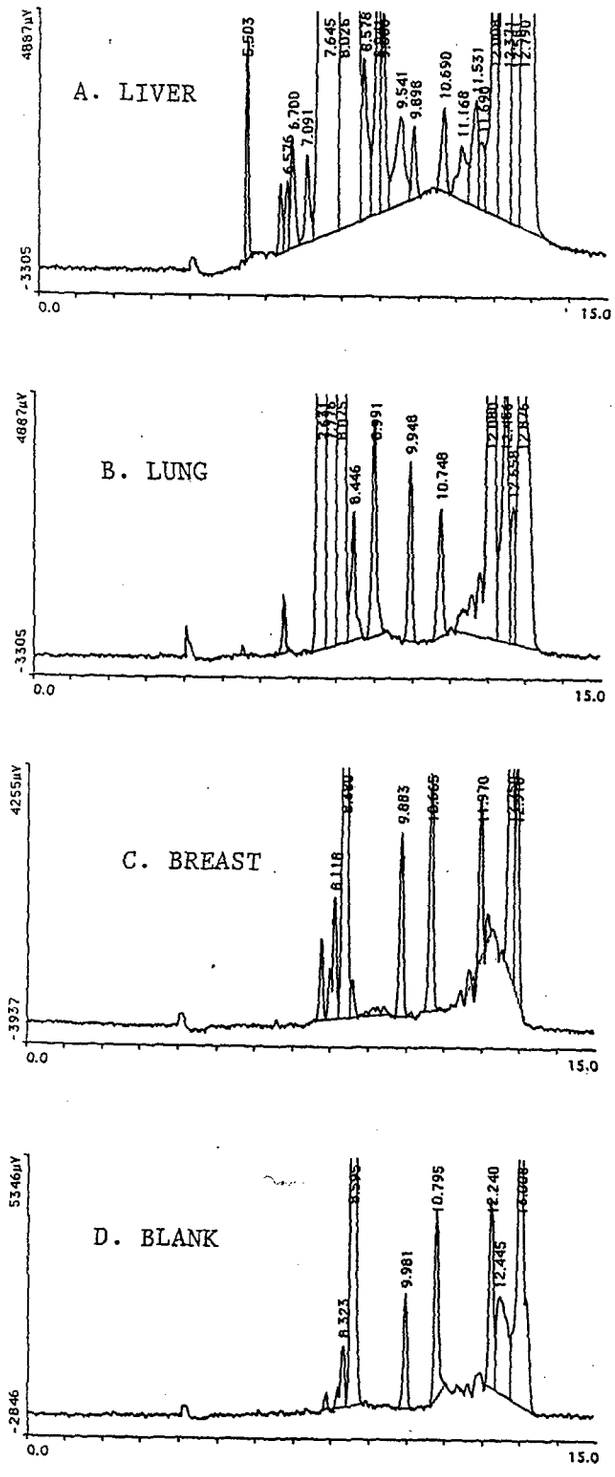


Figure 3

Figure 4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10.690
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11.531
B. LUNG
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15.0
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6.991
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10.748
C. BREAST
4255μV
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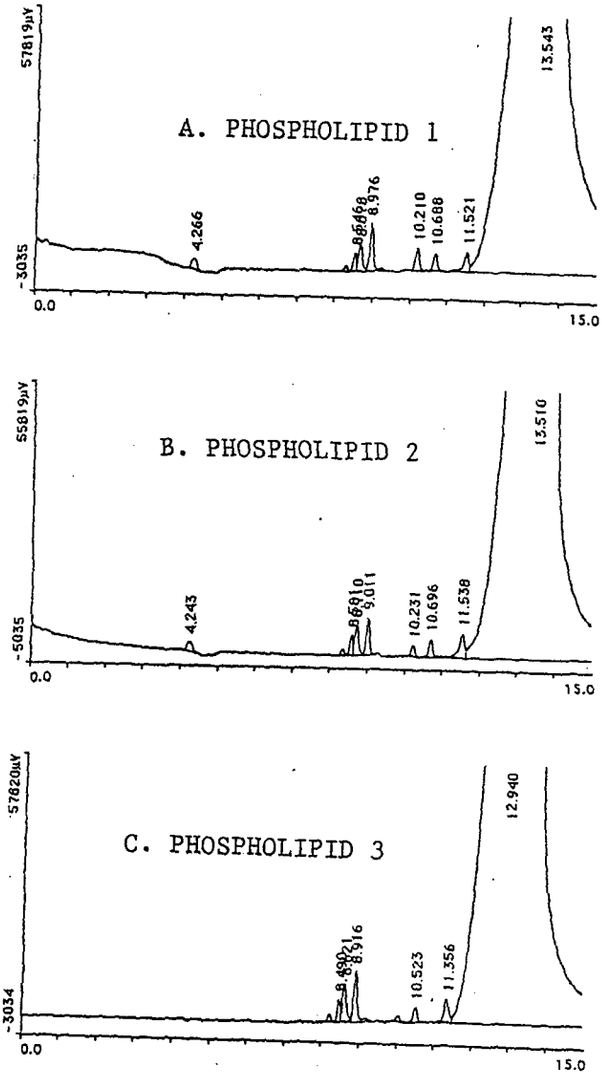
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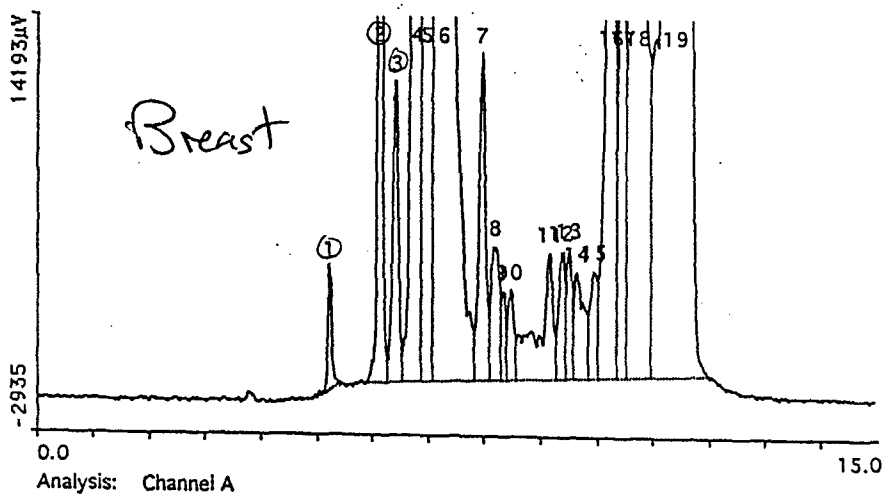
Figure 5



Analysis: Channel A

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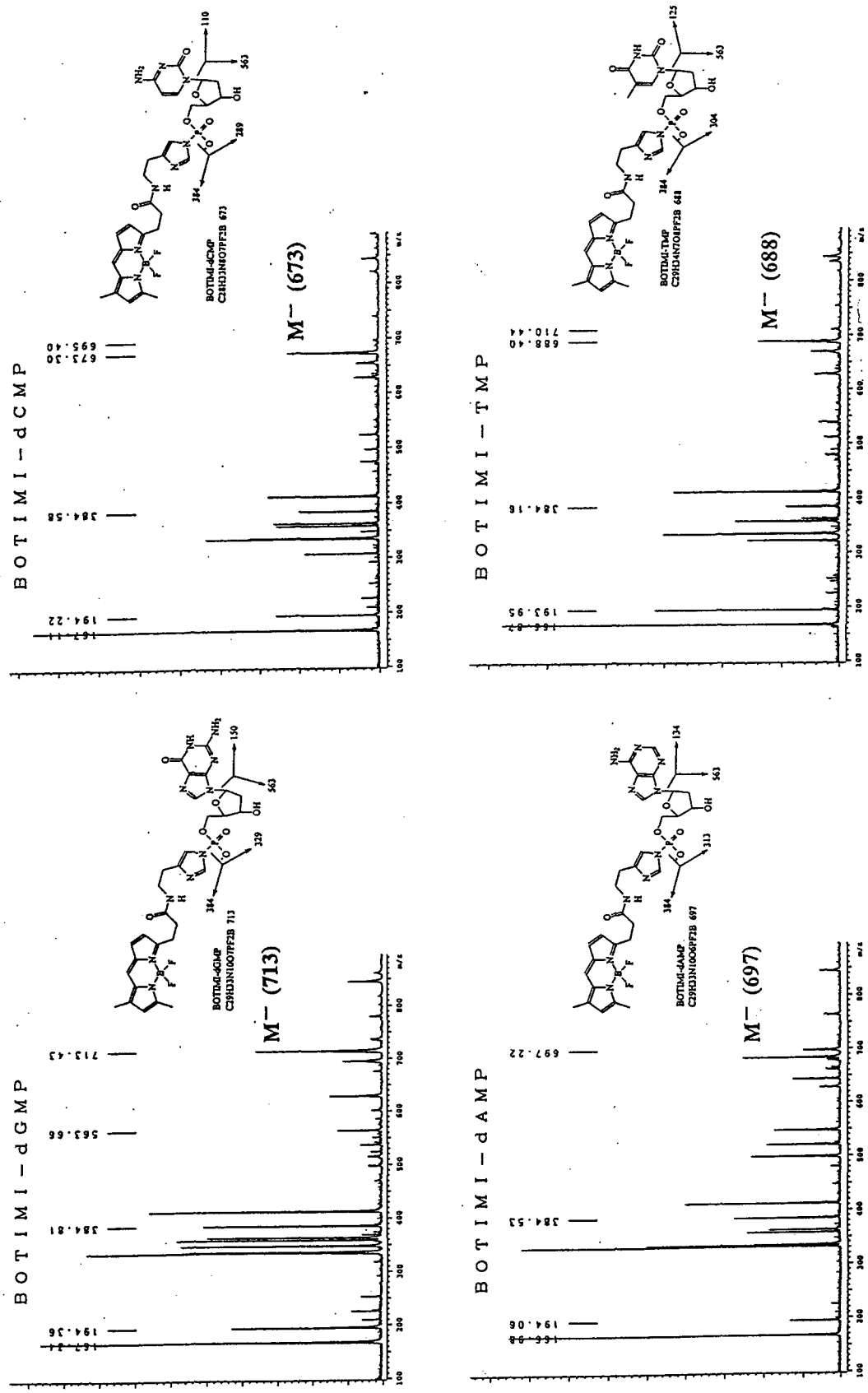
3	6.421	N2	12480	68195	0.190
4	6.808	N3	658823	2809467	7.833
5	6.960	N4	894350	4626332	12.899
6	7.266	Err!	1196219	18625654	51.934
7	7.963	N6	13536	87667	0.244
8	8.183	N7	5503	55270	0.154
9	8.333	N8	3703	18354	0.051
10	8.475	N9	3731	24561	0.068

Figure 6


BOTIMI-dCMP
M⁻ (673)
BOTIMI-TMP
M⁻ (688)
BOTIMI-dGMP
M⁻ (713)
BOTIMI-dAMP
M⁻ (697)

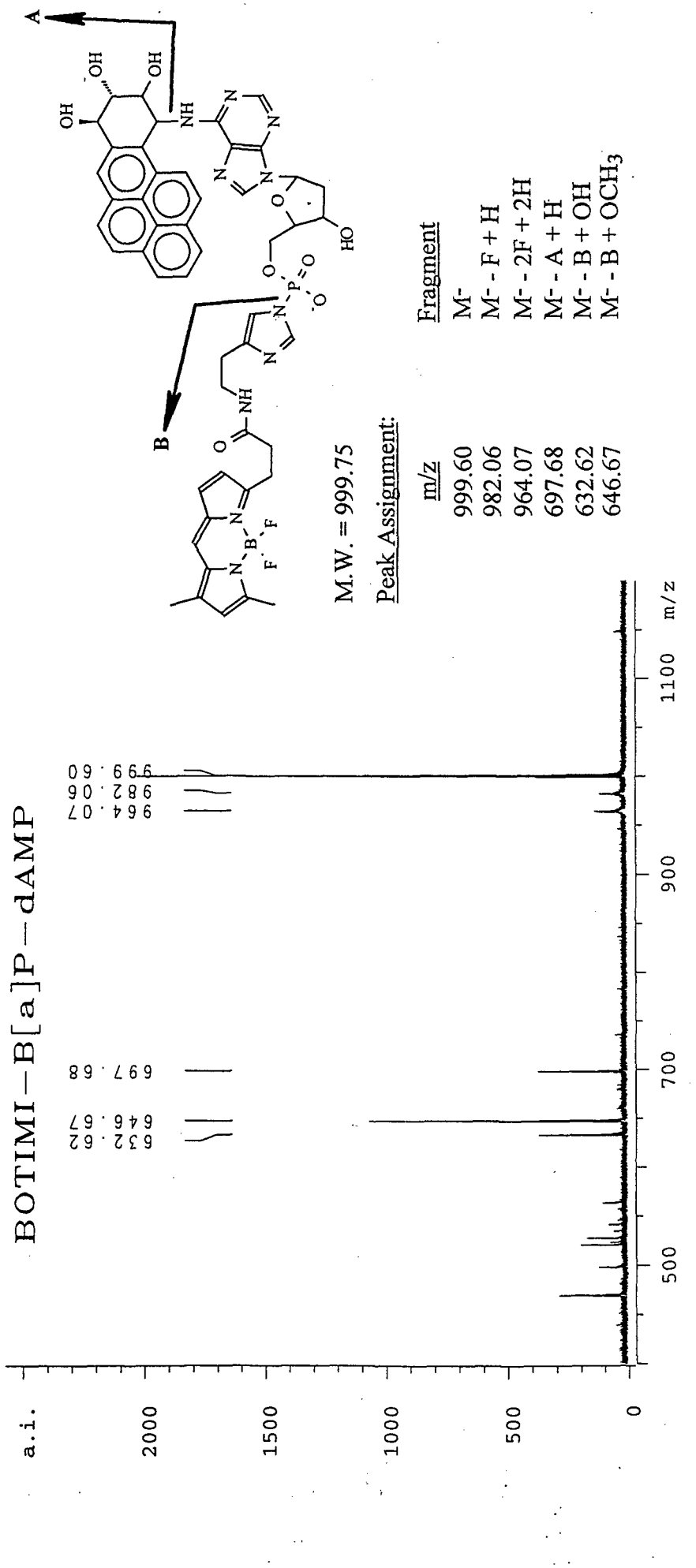

BOTIMI-B[a]P-dAMP
a.i.
2000
1500
1000
500
0
500
700
900
1100
m/z
999.60
982.06
964.07
697.68
646.67
632.62
A
B
M.W. = 999.75
Peak Assignment:
m/z
Fragment
999.60
M-
982.06
M- - F + H
964.07
M- - 2F + 2H
697.68
M- - A + H
632.62
M- - B + OH
646.67
M- - B + OCH3

Figure 7

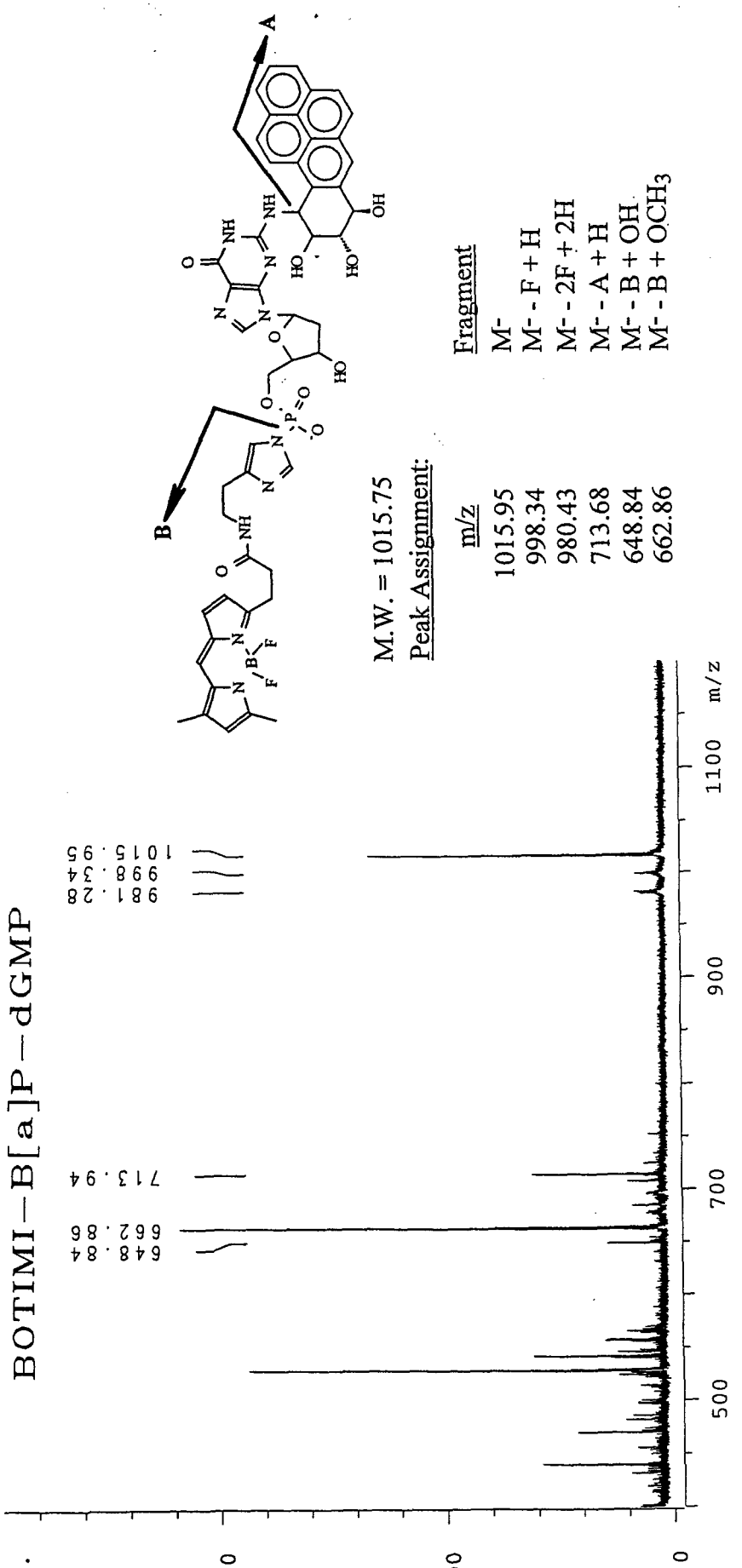
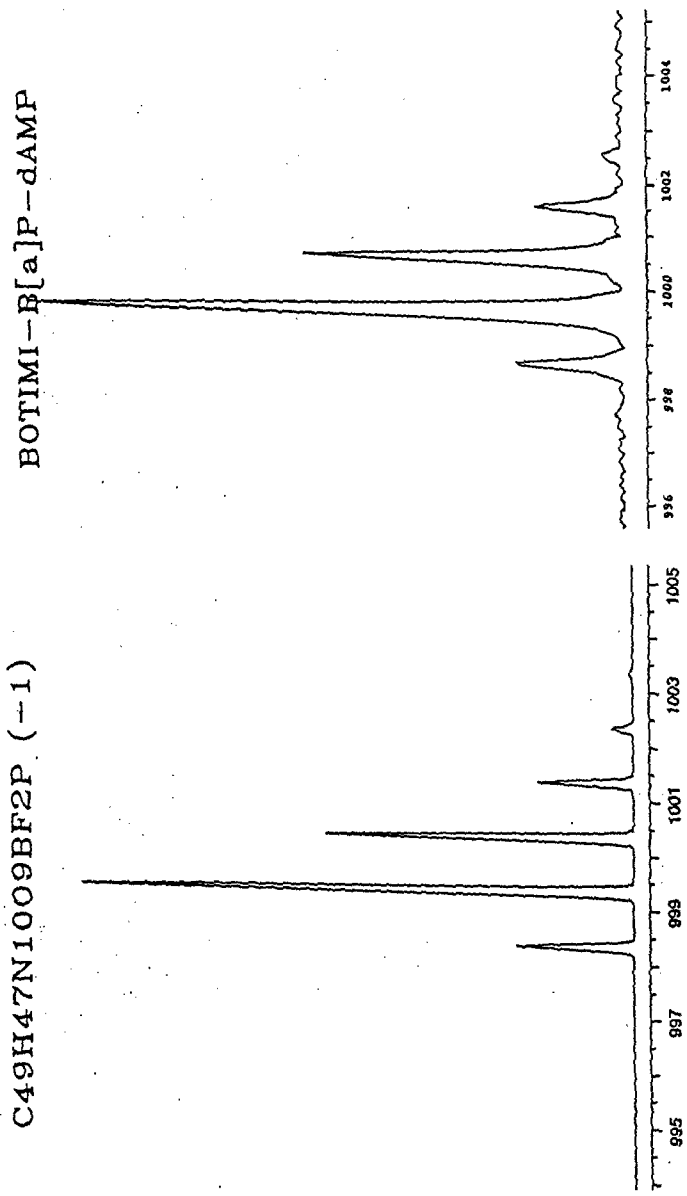

BOTIMI-B[a]P-dGMP
a.i.
1000
500
0
500
700
900
1100
m/z
981.28
998.34
1015.95
648.84
662.86
713.94
A
B
M.W. = 1015.75
Peak Assignment:
m/z
Fragment
1015.95
M-
998.34
M- - F + H
980.43
M- - 2F + 2H
713.68
M- - A + H
648.84
M- - B + OH
662.86
M- - B + OCH3

Figure 8

Figure 9


BOTIMI-B[a]P-dAMP
996
998
1000
1002
1004
C49H47N1009BF2P (-1)
995
997
999
1001
1003
1005

Rec'd
7/19/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 6 Jul 00

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2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at Virginia.Miller@det.amedd.army.mil.

FOR THE COMMANDER:

Phyllis Rinehart

PHYLLIS M. RINEHART
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